Longevity of microorganisms in natural environments

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Bacterial endospores are exceptional among living systems in respect to their resistance to adverse environmental conditions, notably to heat and des~~s~~iccation, and consequently might be expected to show cryptobiosis of very long duration. This report considers recent observations indicating that the maximal longevity of bacterial spores is probably much greater than previously believed.

Introduction

According to Keilin,[1] speculations and observations on 'latent life' originated with van Leeuwenhoek in 1702. In his remarkable 1959 essay on the subject, Keilin discussed various terms used to describe 'latent life' and proposed *cryptobiosis* to describe 'the state of an organism when it shows no visible signs of life and when its metabolic activity becomes hardly measurable, or comes reversibly to a standstill.' What is the maxium duration of cryptobiosis? Obviously, this would be expected to vary considerably, depending on the organism. Keilin's critical assessment of claims in this connection is of great interest, and included observations on lotus seeds in some detail. From 1959, the evidence for viability of such seeds for at least 800 years was convincing, and this has been substantiated by more recent experiments.[2]

During the 1920s and 1930s a number of reports in the literature claimed demonstration of 'amazing longevity' of microorganisms within very ancient natural materials such as coal and Precambrian rocks. These extravagant claims were perceived to be based on faulty bacteriological technique, and were generally discounted (see, for example, reference 3). In 1962, Sneath[4] summarized observations relating to longevity of bacterial spores, and noted that the longest survivals known at that time were 68 years for anthrax spores prepared by Pasteur, and 'spores of a thermophilic bacillus in a tin of meat which had been canned 118 years previously.' These and other reports led Sneath to make tests for viable bacilli in soil particles adhering to roots of plants that were gathered, dried, and stored in the Herbarium of the Royal Botanical Gardens, Kew (Surrey, UK). His experiments clearly showed the presence of viable *Bacillus* spores in samples as old as 320 years. Overall, the longevity of spores of different species paralleled the heat-resistance properties of the respective spore types. Sneath also noted that the death-rate curve of bacteria in the stored samples was similar to the decay curve of a mixture of radioactive isotopes of different half-lives, and estimated that a ton of dry soil would still contain a few viable spores even after 1000 years.

Bacillus spores in ocean sediments

Bartholomew & Paik[5] examined sediments from ocean basin cores for the presence of bacteria, and detected thermophilic bacilli in virtually all samples (from 5 to 900 organisms g^{-1} of wet sediment). Since sea-water sample controls did not contain thermophilic bacilli, it was evident that 'the organisms found in the cores were not due to contamination during the retrieval of the cores from the ocean depths.' From ^{14}C dating results, sediment at the 150 cm core depth was calculated to be at least 5800 years old; such samples contained 25–75 bacilli g^{-1} of wet sediment. Thus, it would appear that thermophilic bacilli can survive, presumably in the form of spores, for thousands of years in cold (*ca.* 4°C), dark, and wet environments. This possibility evidently surprised Bartholomew & Paik, who concluded: 'It is possible that 'alien' sporeforming bacteria may have persisted in such sediments for these long periods of time. However, the burden of proof would fall on anyone who attempted to make such a statement, since this would imply bacterial spore ages of many thousands of years.'

Cryptobiosis of *Thermoactinomyces* spores

Bacteria of the genus *Thermoactinomyces* differ from other actinomycetes in that they form heat-resistant endospores and usually show an elevated optimum growth temperature of about 50°C.[6] These organisms provide excellent subjects for study of spore longevity because of their ubiquity and other properties. Thus: 'Thermophilic actinomycetes can be isolated from most soil samples and they probably proliferate in plant litter and at the soil surface where temperatures in excess of 30°C can be attained as a result of associated microbial activities or from sunlight. High numbers of spores are produced where vegetation is stored, e.g. haystacks and baled bagasse, or when cereals are harvested and stored for subsequent use.'[7]

Investigations by T. Cross and his colleagues[7–9] have provided evidence for viability of *Thermoactinomyces* endospores for time periods of 1900 to 2700 years. Occupational debris from a Roman archaeological site at Vindolanda, Northumberland (UK) was accurately dated and examined for viable spores.[7,9] Colony counts as high as 10^4 per g dry weight were observed in debris

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deposited *ca.* 1900 years ago. The occupational debris, rich in organic matter, was sandwiched between compacted layers of clay, and the preservation of spore viability presumably was enhanced by anaerobic and other favourable chemical and physical conditions. It is noteworthy that internal controls included the observation that the occupational layers showed high *Thermoactinomyces* counts as compared to intervening compacted clay layers.

Ancient viable endospores of *Thermoactinomyces* have also been detected in lake beds in which the chronological sequence of sediment deposition could be dated by one or more techniques. Such experiments on lakes of the English Lake District and an ancient lake bed in East Anglia[7] indicated the presence of viable *Thermoactinomyces* spores in sediments deposited 1900 to 2700 years ago. Additional evidence for remarkable longevity of *Thermoactinomyces* spores comes from study of a 20-m sediment core obtained from Elk Lake, Minnesota, USA in 1978. The lake bed shows a 10 359 year record of sedimentation, clearly revealed by ^{14}C analysis and varve (annual lamination) chronology. Parduhn & Watterson[10] examined core samples for viable *Thermoactinomyces* and a major peak of 'colony forming units' (*ca.* 1000 per gram) was found associated with a layer 11.15 m below the lake bottom, dated as 5144 varve years B.P. (Before Present); smaller peaks were observed at 4392, 6341, and 7173 years B.P.

Natural radiations and spore survival

Sneath[4] noted that one of the uncertain factors in estimating survival of bacterial spores over very long periods of time is the accumulated effect of ionizing radiations. The lethality of natural radiations obviously would be influenced by the presence or absence of protective materials. Sneath's calculations indicated that spores protected from direct radiations by a few centimetres of material would not suffer appreciable radiation damage over long time periods. Similarly, the radiation effects of ^{40}K were estimated to be negligible, leading Sneath to conclude that 'life could probably be preserved for periods of more than a million years, if suitably protected and maintained at temperatures close to absolute zero.' Recent investigations bolster Sneath's assessments. Studies on inactivation of *Bacillus subtilis* spores by ultraviolet radiation in simulated interstellar space environments[11] (ultra-high vacuum and low temperature) suggest that suitable attenuation of ultraviolet flux could permit survival times of the order of millions of years (for example, attenuation by 'dark clouds' or if the spore is coated with 0.9 μm of material with an imaginary index of refraction of 0.5). ^{40}K (half-life, 1.28×10^9 years) is the largest single contributor to the background radiation dose in living organisms, and a recent study[12] demonstrated that this naturally occurring isotope does not make a significant contribution to spontaneous mutation rate in *Escherichia coli.*

We recently undertook a more direct approach to assessment of the accumulation of lethal mutations caused by background radiation in stored spores of *Bacillus subtilis*. The experimental procedure was based on determination of the number of auxotrophic mutants present in an aqueous suspension of spores that had been stored at 4°C for 16 years. In order to estimate the *total* gene damage accumulated, a correction factor was calculated as follows. The *B. subtilis* chromosome contains enough DNA to code for about 6000 genes.[13] Some of the DNA, however, is 'spacer' material, and mutation of some genes that code for inessential proteins will have no effect on viability (e.g. inducible enzymes, flagellar proteins, exo-enzymes etc.). Assuming that roughly 25% of the chromosomal DNA may be dispensable, the number of genes essential for cell viability in a minimal medium should be about 4500. The number of enzymes needed for biosynthesis of amino acids and nucleotides is about 110, but the number of genes concerned will be greater because: (a) many enzymes having different subunits are coded for by more than one gene, and (b) the estimate of 110 does not include enzymes needed for the synthesis of growth factors. Allowing for (a) and (b), it can be assumed that the number of genes required for basic biosynthetic functions is of the order of 200, i.e. about 5% of the functional genome. Thus, the number of auxotrophic mutants found should be multiplied by about 20 to give an estimate of the total gene damage accumulated.

A sizeable number of spores was plated on a rich medium (containing amino acids, purines, pyrimidines, growth factors etc.) in order to give well separated colonies (about 50 per plate). A total of 9960 colonies were grown, and these were replicated onto lactate-minimal medium to determine how many represented auxotrophic mutants that could be considered to be 'operationally dead'. Five of the 9960 failed to grow in the minimal medium. On the basis of the reasoning set out above we can deduce that the mutation rate has been about 5×20 mutations/10000 cells/16 years = 10 mutations/1000 cells/16 years. To know whether this is of any significance, the value calculated must be compared with that obtained for the incidence of auxotrophs in the progeny of fresh spores. Spores from a colony freshly grown on minimal medium, and therefore prototrophic, were plated on nutrient-rich agar (as above) and the resultant colonies were replicated on lactate-minimal medium. The total number of colonies that grew was 9929 and of these, five had developed auxotrophic requirements and consequently failed to grow on the minimal agar. (This is roughly the number that would be expected for a spontaneous mutation rate of about $1:10^7$ cells per gene.) In other words, there was no detectable difference between the number of auxotrophic mutants found in the progeny of fresh spores and in the progeny of those that had been stored for 16 years. Thus, the effects of natural radiation do not appear to be a significant factor in determining the longevity of bacterial spores. Even if the assumption is made that all the mutations found in the 16-year-old suspension had resulted from radiation effects and that fresh spore suspensions contain no auxotrophic mutations, the calculated half-life of the stored spore population would still be about 7000 years. Using this value, and assuming an exponential rate for death resulting from radiation damage, it can be estimated that a population containing 10^{10} spores initially would have a measurable number still viable after 200000 years. If one takes into account the fact that the fresh spore suspension showed about the same incidence of auxotrophs, it is apparent that the duration of viability might well be longer, possibly even by some orders of magnitude. This is in keeping with Sneath's estimate already referred to.

Long-term stability of biological macromolecules

The maximum duration of cryptobiosis evidently will be determined by the stability of an assortment of macromolecules that include, primarily, nucleic acids and proteins. Keilin[1] cites studies in which stability of blood-group proteins for *ca.* 5000 years was indicated by serological reactivity. More recently, haemoglobin (or antigenic fragments thereof) was detected in 4500-year-old bones.[14] Other reports describe immunological detection of albumin in 40000-year-old mammoth tissue[15] and collagen in even older specimens.[16] We are not aware of attempts to demonstrate survival of enzymatic activities in well preserved biological samples of great antiquity, but there seems no reason to doubt a priori that enzymes and other proteins might remain intact for long periods under appropriate conditions. Similarly, there is every reason to believe that nucleic acids could maintain biological integrity for many millenia: this view is supported by the recent molecular cloning of DNA from a 2400-year-old Egyptian mummy.[17]

It appears that the stability of proteins, nucleic acids and other macromolecules required for maintenance of viability for thousands of years would be maximized by anhydrous conditions. The resistance of bacterial spores to adverse conditions is generally attributed to the fact that the core of the spore protoplast is essentially dry.[18,19] Ordinary vegetative cells of *Bacillus* spp. have a water content of approximately 80%, whereas the central protoplast portion of *Bacillus* spores has only about 20%.[19]

Concluding remarks

Since background radiation apparently can be ignored as a significant factor affecting longevity of spores on a time scale extending into many thousands of years, it is not surprising that there are reports of spores surviving for periods of 2000 to 7000 years in archaelogical sites and mud cores from lake beds and ocean basins. Thus, the maximal longevity of bacterial spores seems to be much greater than is commonly supposed, and we conclude that renewed efforts to detect viable spores of greater antiquity is a feasible and worthwhile endeavour. Careful selection of materials to be examined and application of new experimental techniques should eliminate the uncertainties that undermined the veracity of earlier attempts to establish the limits of cryptobiosis of bacterial spores.

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